

THE NATURE OF THE ANTIBIOTIC SUBSTANCES PRODUCED BY *ASPERGILLUS FUMIGATUS*^{1,2}

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INTRODUCTORY

The formation of more than one antibiotic substance by a single antagonistic organism has been definitely established. *Penicillium notatum*, for example, has been shown to produce penicillin and notatin; and *Aspergillus flavus*, aspergillic acid and flavicin. The ability of different microorganisms to produce the same, or a closely related, antibiotic substance has also been demonstrated, as illustrated by the fact that penicillin and flavicin, two of the substances just mentioned as being produced by two distinctly different microorganisms, are similar, if not identical, chemically as well as biologically. Another and even more striking illustration is offered, in this connection, by *Aspergillus fumigatus*. This organism has now been shown to produce four distinctly different antibiotic substances, two of which are closely related chemically.

Oxford and Raistrick (1942) found that certain strains of *A. fumigatus* are able to produce spinulosin and fumigatin, pigmented quinones, both of which possess antibacterial properties. The first possesses only weak antibiotic activity; the second is more active, being capable of bringing about the complete inhibition of growth of certain strains of *Staphylococcus aureus* in dilutions of 1:50,000. The bacteriostatic action of fumigatin against *Escherichia coli* is very limited, however. Waksman, Horning, and Spencer (1943) isolated from the culture filtrate of *A. fumigatus* grown in a simple synthetic medium a colorless easily crystallizable compound that showed much greater antibacterial activity than fumigatin; this substance, designated as fumigacin, was active largely against various gram-positive bacteria, which were inhibited in a dilution of 1:750,000 to 1:4,000,000; gram-negative bacteria were only slightly affected. Fumigacin contained a small amount of nitrogen and showed some toxicity to animals.

Recently, Menzel, Wintersteiner, and Hoogerheide (1944) demonstrated that *A. fumigatus* produces a fourth compound, namely gliotoxin, a substance that previously was isolated from certain strains of *Trichoderma* and *Gliocladium* by Weindling (Weindling and Emerson, 1936). Gliotoxin contains 8.59 per cent nitrogen and 19.65 per cent sulfur. These investigators demonstrated that the crystalline preparation of fumigacin obtained by Waksman, Horning, and Spencer (1943) contains about 20 per cent of a gliotoxin fraction. This accom-

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panying fraction accounted for the sulfur and nitrogen present in the fumigacin. The gliotoxin fraction was also found to be largely responsible for the toxicity of the fumigacin. When the fumigacin was freed from the accompanying gliotoxin it contained no nitrogen and was given the tentative formula of $C_{32}H_{44}O_8$; it was also much less toxic than the fumigacin prepared by Waksman, Horning and Spencer (1943). Unaware of these findings, Chain, Florey, Jennings, and Williams (1943) isolated from *A. fumigatus* a crystalline preparation, designated as helvolic acid, that is apparently identical with the fumigacin from which the gliotoxin fraction has been removed, as shown by chemical and biological, as well as by *in vivo* activity.

The following results are presented in order to confirm and extend the findings of Menzel, Wintersteiner, and Hoogerheide (1944) and to establish the relation between the formation of fumigacin and the other antibiotic substances by *A. fumigatus*.

EXPERIMENTAL

In the survey made by Waksman and Horning (1943) on the distribution of antibiotic properties among fungi, it was reported that 15 different strains of *A. fumigatus* were isolated. These cultures varied greatly in their antibiotic activity and in the amount of fumigacin produced. One culture, No. 84, was largely used in these investigations.

The method of extraction of fumigacin from the medium was found to have a considerable influence upon the purity of the fumigacin obtained and upon the amount of gliotoxin accompanying it. When the medium is treated by the WHS method (Waksman, Horning, and Spencer, 1943), namely, adsorption on norit, followed by extraction with chloroform, or ether followed by chloroform, only a limited amount of gliotoxin is found admixed with the fumigacin. However, where the two substances are extracted directly from the medium, using the MWH method (Menzel, Wintersteiner, and Hoogerheide, 1944) a much larger yield of gliotoxin is obtained, the yield of fumigacin remaining, however, practically the same.

A quantity of crystalline fumigacin obtained by the WHS method was further purified by means of the MWH method with the following results, the antibacterial activity being expressed in terms of *S. aureus* units:

		Activity
Fumigacin crystals (WHS).....	443 mg	1,000,000
Pure fumigacin (MWH).....	343 mg	1,000,000
Gliotoxin fraction.....	89 mg	100,000

These results show that the fumigacin prepared by the WHS method contains about 20 per cent of a gliotoxin fraction and about 80 per cent of purified fumigacin. The gliotoxin fraction had little antibacterial action, which was apparently lost during the chemical treatment, this compound being highly labile. Virtually all the activity was found in the purified fumigacin.

In order to isolate the various antibiotic substances, *A. fumigatus* was grown upon a Czapek-Dox tap water medium for 7 days at 28°C. The culture filtrate

was divided into two lots; each was treated by the two different methods and the several antibiotic substances were isolated (table 1). When the medium was extracted directly (MWH method), nearly five times as much gliotoxin was produced as fumigacin. The WHS method gave the same yield of fumigacin but a much lower yield of gliotoxin. The difference in the relative yields of the two antibiotic substances by the WHS method, as shown by the results in the above summary and in table 1, is no doubt due to the fact that the earlier lot of fumigacin was recrystallized several times, which tended to reduce or destroy the gliotoxin fraction (fig. 1).

Based upon the results of Oxford and Raistrick (1942) and of Menzel, Wintersteiner, and Hoogerheide (1944), the chemical properties of the four antibiotic substances produced by *A. fumigatus* are summarized in table 2. In view of the fact that spinulosin is a derivative of fumigatin and is produced only by certain strains of *A. fumigatus* and especially since it is only a weak antibiotic agent, it can be left out of further consideration. The other three antibiotic

TABLE 1
Yields of different antibiotic substances by different methods of extraction
Per liter of medium

	WHS METHOD*	MWH METHOD†
	mg	mg
Fumigatin.....		39.3
Fumigacin.....	15.1	17.8
Gliotoxin.....	27.1	85.8

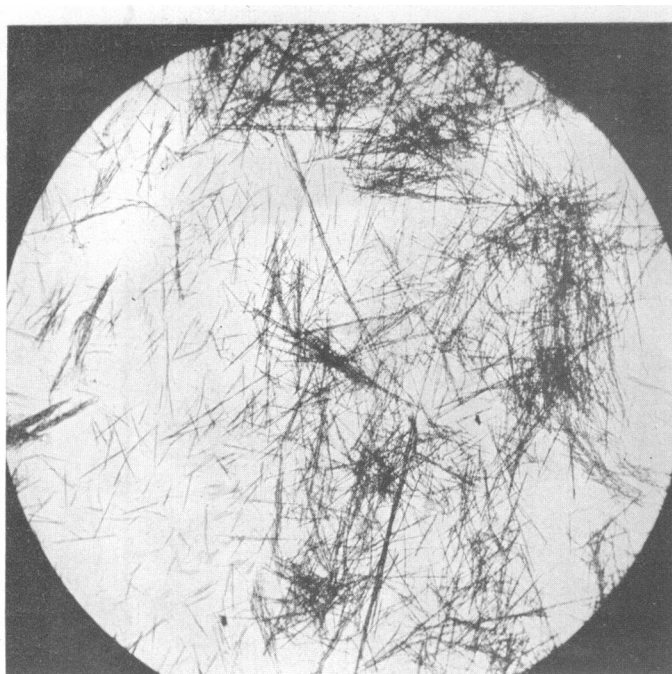
* Adsorption on norit; treatment with ether, followed by chloroform; crystallization from alcohol.

† Extraction of acidified medium with ether, removing pigment with NaHCO_3 solution, and fumigacin in 6% Na_2CO_3 solution.

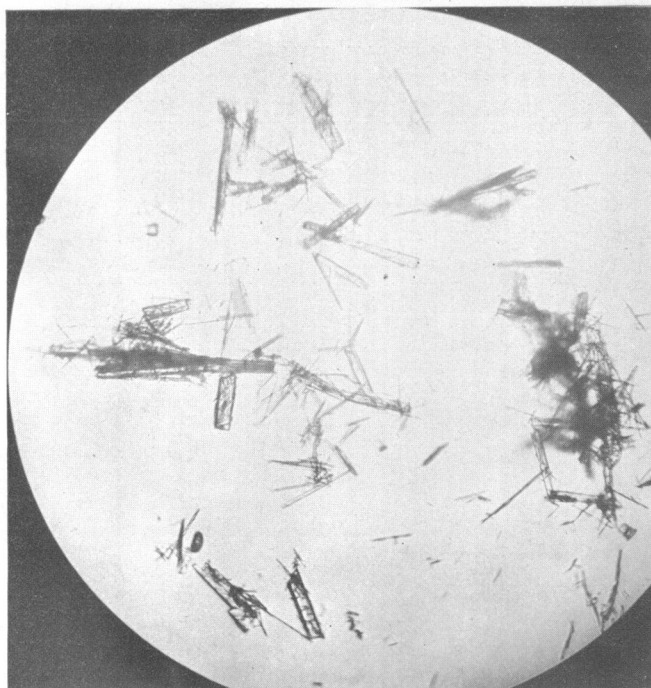
substances were next isolated from a single lot of culture filtrate of *A. fumigatus* No. 84, and their antibacterial properties were measured by the use of the agar-plate dilution method. The results are reported in table 3. Of the three compounds produced by *A. fumigatus*, fumigatin proved to be, as one might have expected from the results of Oxford and Raistrick (1942), the least active antibacterial agent. Gliotoxin was more active than fumigacin, against both the gram-negative *E. coli* and the gram-positive *Bacillus subtilis* and *Sarcina lutea*.

It may be of interest to compare the results of the antibacterial activity of gliotoxin isolated from *A. fumigatus* with those reported by Johnson, Bruce, and Dutcher (1943) for gliotoxin produced by *Gliocladium*. The antibiotic activity of the latter was reported to be 10,000 *E. coli* units and 3,000,000 *S. lutea* units. These results are quite comparable, especially if one keeps in mind the fact that different methods were used in making the tests and that the gliotoxin molecule is very unstable.

Finally, an experiment was conducted to check the finding of Menzel, Wintersteiner, and Hoogerheide (1944) that better yields of fumigacin were obtained



I



II

FIG. 1. CRYSTALS OF COLORLESS ANTIBIOTIC SUBSTANCES PRODUCED BY *ASPERGILLUS FUMIGATUS*. I. FUMIGACIN. II. GLIOTOXIN

from the medium when distilled water replaced tap water. The results reported in table 4 show that, contrary to the above results, the tap water medium gave highest yields of all three antibiotic substances, and that the substitution of the

TABLE 2
Chemical properties of four antibiotic substances produced by A. fumigatus

	SPINULOSIN	FUMIGATIN	FUMIGACIN	GLIOTOXIN
Crystallization.....	Purplish-bronze plates	Maroon-colored needles	Very fine white needles	Elongated plates
M.P.....	201°C	116°C	215–220°C	195°C
Formula.....	C ₈ H ₈ O ₆	C ₈ H ₈ O ₄	C ₃₂ H ₄₄ O ₈ *	C ₁₁ H ₁₄ O ₄ N ₂ S ₂ †

* Chain, Florey, Jennings and Williams (1943) reported for the helvolic acid the same formula.

† Johnson, Bruce and Dutcher (1943).

TABLE 3
Bacteriostatic potency of three antibiotic substances produced by A. fumigatus
Dilution units by agar streak method

ORGANISM	FUMIGATIN	FUMIGACIN	GLIOTOXIN
<i>E. coli</i>	1,200	1,200	6,000
<i>S. aureus</i>	200,000	2,000,000	1,500,000
<i>B. subtilis</i>	40,000	100,000	750,000
<i>S. lutea</i>	100,000	1,000,000	2,000,000

TABLE 4
Effect of nature of water and sulfate content of medium upon the production of the various antibiotic substances by A. fumigatus

	DISTILLED WATER				TAP WATER			
	Sulfates*		Chlorides		Sulfates		Chlorides	
	Yield†	Activity‡	Yield	Activity	Yield	Activity	Yield	Activity
	mg	units	mg	units	mg	units	mg	units
Culture filtrate		100		<10		100		10
Fumigatin.....	41	100,000	0		205	100,000	43	200,000
Fumigacin.....	31	800,000	3	80,000	47	800,000	18	800,000
Gliotoxin.....	106	1,500,000	23	80,000	137	300,000	36	20,000

* Magnesium (0.5 gm per liter) and iron (0.01 gm per liter) salts in medium in form of sulfates or chlorides.

† On basis of 2 liters of culture medium.

‡ Dilution units, with *S. aureus* as test organism.

sulfates by chlorides led to the virtual elimination of the substances in the distilled water medium and to a considerable reduction in the tap water medium. The activity of the gliotoxin fraction isolated from the chloride-containing media

was very much lower than that of the corresponding fraction in the sulfate media; this points to the fact that the fraction is not a typical gliotoxin at all and may represent a small amount of fumigacin in solution, or some other antibiotic constituent. This was confirmed by the observation that the two fractions of gliotoxin obtained from sulfate-containing media contained sulfur whereas the two others did not.

The previous findings on the toxicity of fumigacin, as reported by Waksman, Horning and Spencer (1943) and by Robinson (1943) must now be considered in a new light, namely in terms of purified fumigacin, freed from the gliotoxin fraction. Whereas earlier reports indicated that 4 mg of fumigacin injected into 20 gm weight mice by the intraperitoneal method showed evidence of toxicity, the purified fumigacin was tolerated by mice even in concentrations of 16 mg/20 gm. These results are in full conformity with those reported by Menzel, Wintersteiner and Hoogerheide (1944) and by Chain, Florey, Jennings and Williams (1943).

SUMMARY

Aspergillus fumigatus represents a type of antagonistic organism that produces several antibiotic substances. These differ in their chemical nature and in the range of their antibacterial action, or their antibiotic spectra. Of the three substances produced by this organism, namely, fumigatin (another compound, spinulosin, is chemically related to fumigatin and is produced only by certain strains of this organism), fumigacin and gliotoxin, the first is the least active; fumigacin is more active, and gliotoxin is the most active. Gliotoxin also acts upon a greater number of bacteria than fumigacin, including various gram-negative bacteria. Gliotoxin is more toxic to animals than fumigacin. A compound recently described by British investigators as helvolic acid is apparently the same as fumigacin.

Of the three compounds produced by *A. fumigatus*, fumigacin, because of its lower toxicity to animals and its *in vivo* activity, offers the greatest promise as a chemotherapeutic agent. It is far less active, however, than penicillin.

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